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Amino Acid Residues 24–31 but not Palmitoylation of Cysteines 30 and 45 Are Required for Membrane Anchoring of Glutamic Acid Decarboxylase, GAD_{65}

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Abstract. The smaller isoform of the GABA synthesizing enzyme glutamic acid decarboxylase, GAD_{65}, is synthesized as a soluble protein that undergoes posttranslational modification(s) in the NH_2-terminal region to become anchored to the membrane of small synaptic-like microvesicles in pancreatic β cells, and synaptic vesicles in GABA-ergic neurons. A soluble hydrophilic form, a soluble hydrophobic form, and a hydrophobic firmly membrane-anchored form have been detected in β cells. A reversible and hydroxylamine sensitive palmitoylation has been shown to distinguish the firmly membrane-anchored form from the soluble yet hydrophobic form, suggesting that palmitoylation of cysteines in the NH_2-terminal region is involved in membrane anchoring. In this study we use site-directed mutagenesis to identify the first two cysteines in the NH_2-terminal region, Cys 30 and Cys 45, as the sites of palmitoylation of the GAD_{65} molecule. Mutation of Cys 30 and Cys 45 to Ala results in a loss of palmitoylation but does not significantly alter membrane association of GAD_{65} in COS-7 cells. Deletion of the first 23 amino acids at the NH_2 terminus of the GAD_{65}30/45A mutant also does not affect the hydrophobicity and membrane anchoring of the GAD_{65} protein. However, deletion of an additional eight amino acids at the NH_2 terminus results in a protein which is hydrophilic and cytosolic. The results suggest that amino acids 24–31 are required for hydrophobic modification and/or targeting of GAD_{65} to membrane compartments, whereas palmitoylation of Cys 30 and Cys 45 may rather serve to orient or fold the protein at synaptic vesicle membranes.

FLEXIBLE membrane association is an important feature of several proteins involved in membrane trafficking and intracellular signaling (Fischer et al., 1990; Magee et al., 1987; Pate Skene and Virág, 1989; Levis and Bourne, 1992). It is also a characteristic of some enzymes involved in the biosynthesis of neurotransmitters. Thus glutamic acid decarboxylase, the smaller form of the γ-amino butyric acid (GABA) synthesizing enzyme glutamic acid decarboxylase, GAD_{65},[1] (Christgau et al., 1991, 1992), tyrosine hydroxylase, the initial and rate limiting enzyme in the synthesis of catecholamines (Kuhn et al., 1990), and dopamine β-hydroxylase, the final enzyme in the synthesis of norepinephrine (Bon et al., 1991), share flexible membrane anchoring to secretory vesicles that store and release their neurotransmitter, suggesting an important regulatory role of membrane localization.

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1. *Abbreviations used in this paper*: aa, amino acid; GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase; MHC, major histocompatibility complex; PLP, pyridoxal 5'-phosphate; wt, wild-type.

GAD is encoded for by two distinct non-allelic genes, GAD_{67} and GAD_{65}, which are probably derived from a common ancestor gene and share significant homology except in the first 95 amino acids (Erlander et al., 1991). Both proteins are synthesized in the cytoplasm as hydrophilic and soluble molecules, and GAD_{67} remains in this form. GAD_{65} is posttranslationally modified and becomes anchored to the membrane of small synaptic-like microvesicles (Christgau et al., 1991, 1992). The two GAD proteins appear to comprise a dual system for GABA synthesis (Erlander et al., 1991). GAD_{67} is tightly associated with the coenzyme pyridoxal 5'-phosphate (PLP), and may be responsible for constitutive production of GABA. A significant fraction of GAD_{65} is detected as the apoenzyme and is PLP inducible. Thus activation of GAD_{65} can be regulated by PLP levels, and this form may be responsible for generation of GABA in situations of sudden increases in GABA demand (Martin et al., 1991). The membrane localization of GAD_{65} may facilitate delivery of GABA directly to a synaptic vesicle or synaptic like microvesicle for rapid accumulation and secretion. In contrast GABA synthesized by GAD_{67}, if destined for regulatory secretion, has to be transported from the cytosol and taken up by synaptic vesicles.

The GAD_{65} protein is unusually susceptible to becoming a target of autoimmunity that affects its major sites of expression, pancreatic β cells and GABA-ergic neurons. Thus GAD_{65} is a major target antigen in both Type 1 diabetes, which results from an autoimmune destruction of β cells (Bækkeskov et al., 1990; Tisch et al., 1993; Kaufman et al., 1993), and in stiff-man syndrome which affects GABA-ergic neurons (Solimena et al., 1990). In contrast, GAD_{67} does not seem to play an independent role as an autoantigen. Rather, GAD_{67} appears to be recognized only when the immune response to GAD_{65} involves cross-reacting epitopes in the two proteins (Velloso et al., 1993; Hagopian et al., 1993; Seißler et al., 1993). We have proposed that the membrane anchoring of GAD_{65} to a secretory vesicle is of significance for the presentation of this form to the immune system and its selective role as an autoantigen (Christgau et al., 1992).

The posttranslational membrane anchoring of GAD_{65} involves at least two steps. The first step is irreversible and results in a hydrophobic molecule which is still mainly localized in the cytosol. The second step is reversible and results in a firm membrane anchoring of the protein (Christgau et al., 1991). The soluble and membrane associated forms of GAD_{65} show identical location on two-dimensional gels, suggesting that they are distinguished by small noncharged lipid moieties (Christgau et al., 1991). The modifications of GAD_{65} do not seem to involve polyisoprenylation, myristoylation, or a phosphatidylinositol glycan. However, palmitoylation distinguishes the membrane-associated and soluble forms of GAD_{65}, suggesting that this fatty acid is involved in membrane anchoring (Christgau et al., 1992). GAD_{65} has a proteolytic hot spot 69–80 amino acids into the molecule. Trypsin cleavage at amino acid (aa) 69 or 70 results in a soluble hydrophilic COOH-terminal fragment and a small membrane bound palmitoylated fragment, demonstrating that the NH_2-terminal region is the site of lipid modification and membrane anchoring of GAD_{65} (Christgau et al., 1992). Consistent with this result, a hybrid molecule containing the NH_2-terminal 1–83 amino acids of the GAD_{65} molecule and amino acids 89–593 from GAD_{67} is membrane associated (Solimena et al., 1993).

In this study we have analyzed the structural requirements of palmitoylation of GAD_{65} and assessed its role in membrane anchoring. Our results suggest that palmitoylation does not play the major role in membrane anchoring of GAD_{65}. Rather a small segment of amino acids adjacent to the palmitoylation sites is required for targeting and membrane anchoring of GAD_{65}.

Materials and Methods

Generation of GAD_{65} Mutants

A 2.4-kb cDNA clone of rat GAD_{65} (a gift from Dr. A. Tobin, University of California, Los Angeles, CA) was used in this study (Erlander and Tobin, 1991). The cDNA carried the entire coding region and 80- and 400-bp 5′ and 3′ untranslated sequences, respectively, and was originally cloned into the EcoRI site of the Bluescript vector (Stratagene, La Jolla, CA). The sequence spanning the first AUG was altered to match the Kozak consensus sequence for optimum translation efficiency (Kozak, 1991) by site directed in vitro mutagenesis. The resulting plasmid was named pBL-YS10. Oligonucleotide-mediated in vitro mutagenesis was carried out according to Kunkel (1987). Sequencing of mutant DNA with Sequenase (Boehringer Mannheim Corp., Indianapolis, IN) was carried out using protocols provided by the manufacturer. The wild-type cDNA with a Kozak sequence was subcloned from pBL-YS10 into the KpnI and NotI sites of the expression vector pSV-SPORT (GIBCO BRL, Gaithersburg, MD). The resulting plasmid was named pSV-YS10 (see Fig. 2, WT). Amino acid substitution mutants were generated by changing Cys to Ala or Ser residues using pBL-YS10 as template DNA. The resulting mutant DNAs were subcloned into the NotI and HindIII sites of the pCDM8 vector (Seed and Aruffo, 1987) or KpnI and XbaI sites of the pSV-SPORT vector. Expression vectors that carry Cys to Ala substitutions were pCDM-YS17 (see Fig. 2 *A*, Cys 30 to Ala, 30A), pCDM-YS35 (see Fig. 2 *A*, Cys 45 to Ala, 45A), and pSV-YS17/35 (see Fig. 2 *A*, Cys 30 and Cys 45 to Ala, 30/45A). The expression vectors that carry Cys to Ser substitutions were pCDM-YS1 (see Fig. 2 *A*, Cys 73 to Ser, 73S), pCDM-YS2 (see Fig. 2 *A*, Cys 75 to Ser, 75S), pCDM-YS3 (see Fig. 2 *A*, Cys 73 and Cys 75 to Ser, 73/75S), pCDM-YS4 (see Fig. 2 *A*, Cys 80 to Ser, 80S), pCDM-YS5 (see Fig. 2 *A*, Cys 82 to Ser, 82S), pCDM-YS6 (see Fig. 2 *A*, Cys 80 and Cys 82 to Ser, 80/82S), pCDM-YS39 (see Fig. 2 *A*, Cys 75, Cys 80, and Cys 82 to Ser, 75/80/82S), and pCDM-YS8 (see Fig. 2 *A*, Cys 101 to Ser, 101S). Plasmid pMT-YS11 carried an insertion of a cDNA fragment lacking the NH_2-terminal 101 amino acids at the EcoRI site of the expression vector pMT2 (a kind gift from R. J. Kaufman, Genetics Institute, Cambridge, MA). The deletion fragment was generated by oligonucleotide-directed mutagenesis by changing the Cys 101 into the first AUG codon flanked by a Kozak consensus sequence (Kozak, 1991) and an EcoRI site 5′ to the consensus sequence.

Expression plasmids for wild-type and mutant $p21^{H\text{-}ras}$ (181/4S) were kindly donated by Dr. J. Hancock (Onyx, Emeryville, CA) (Hancock et al., 1990).

Generation of Deletion Mutants by PCR

Systematic deletions of the amino terminus of the rat GAD_{65} 30/45A mutant were generated by polymerase chain reactions (PCRs) using anchored primers. The 5′ primers carried an overhang containing the Kozak consensus sequence (Kozak, 1991), and the 3′ primers carried an overhang containing a translation termination codon. The 5′ and 3′ primer also carried a KpnI site upstream from the Kozak consensus sequence and an XbaI site downstream of the termination codon to facilitate unidirectional insertion of the amplified expression cassette into the KpnI and XbaI sites of the COS expression vector pSV-SPORT. Plasmid pCDM-YS17/35 was used as template DNA. The high fidelity Pfu thermostable DNA polymerase (Stratagene) was used to minimize the rate of mismatches in the amplification reaction.

Sequencing of the deletion mutants was carried out with an automated sequencing apparatus (Applied Biosystems, Inc., Foster City, CA). The deletion fragments were subcloned into the KpnI and BglII sites of the expression vector pSV-YS10 replacing the first 357 amino acids of GAD_{65} with a deleted NH_2-terminal region. Expression vectors that carry the NH_2-terminal deletions were pSV-YS63, Δ1-8; pSV-YS64, Δ1-15; pSV-YS65, Δ1-23; pSV-YS66, Δ1-31; pSV-YS67, Δ1-38 (Fig. 2 *B*).

Culture of Cells and Transfection

For transient transfection, COS-7 cells were seeded on 60-mm petri dishes and cultured for 15–24 h to 60–70% confluency. The cells were transfected using DOTAP (Boehringer Mannheim Corp.) by adding 100 μl mixture containing 5 μg DNA and 25 μl DOTAP in transfection buffer (20 mM Hepes/NaOH, pH 7.4, 150 mM NaCl). Transfected cells were refreshed after 12–18 h and incubated for additional 48 h before biosynthetic labeling and immunoprecipitation studies or harvesting for subcellular fractionation and/or Triton X-114 partition studies.

Antisera

The antisera used in this study included two rabbit polyclonal antisera, 1266 and 1267, and two mouse monoclonal antibodies, GAD6 and Ab-1. The 1266 and 1267 antisera (a gift from J. S. Petersen, Hagedorn Research Laboratory, Gentofte, Denmark) and the 1701 serum were raised in rabbits against a synthetic peptide containing the last 19 amino acids of the COOH-terminal region of GAD_{67}. These antisera recognize GAD_{65} and GAD_{67} equally well on western blots (Kim et al., 1993). The GAD6 monoclonal antibody (Chang and Gottlieb, 1988) (a gift from Dr. D. Gottlieb, Washington University, St. Louis, MO) is specific for GAD_{65} (Kim et al., 1993). Monoclonal antibody Ab-1 against $p21^{H\text{-}ras}$ was from Oncogene Science Inc. (Uniondale, NY).

Biosynthetic Labeling and Immunoprecipitation

For labeling with [^{35}S]methionine, cells were washed twice in methionine free DME medium and incubated for 4 h in 2.5 ml of the same medium supplemented with 100 μCi [^{35}S]methionine, 1% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For labeling with [^{3}H]palmitic acid, cells (10^6 in a 60-mm petri dish) were washed twice in DME, followed by incubation for 4 h in 2.5 ml of DME supplemented with 0.5 mCi [^{3}H]palmitic acid, 1% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, nonessential amino acids (50 μM alanine, 100 μM each of asparagine, aspartic acid, glutamic acid, glycine, proline, and serine), and 5 mg/ml fatty acid free bovine serum albumin. After labeling, cells were washed once in ice cold DME, then twice in cell harvesting buffer (20 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 10 mM benzamidine/HCl). Washed cells were extracted in 0.5 ml hypotonic Hepes buffer (10 mM Hepes/NaOH, pH 7.4, 1 mM $MgCl_2$, 1 mM aminoethylisothiouronium bromide hydrobromide, 0.2 mM PLP, 1 mM PMSF containing 1% Triton X-114), followed by centrifugation to remove insoluble debris. The soluble extracts (500 μl) were subjected to immunoprecipitation analysis. Immunoprecipitation of labeled GAD_{65} proteins was performed as described previously by Christgau et al., (1991, 1992) using the monoclonal antibody GAD6 for GAD_{65} and its point mutants, and antiserum 1266 for deletion mutant pMT-YS11. Before immunoprecipitation with the 1266 antiserum, the cell extract was supplemented with 0.1% SDS. Immunoprecipitates were analyzed by 10% SDS-PAGE and fluorography (Bækkeskov et al., 1989).

Subcellular Fractionation

Transfected COS 7 cells from a 6-cm petri dish (10^6) were washed twice with phosphate buffered saline and homogenized at 4°C in 0.5 ml of hypotonic Hepes buffer containing 1 mM Na_3VO_4. The homogenate was centrifuged at 800 *g* for 10 min at 4°C to pellet the nuclei. The postnuclear supernatant was centrifuged at 100,000 *g* for 1 h to separate cytosol (S) and particulate fractions. The resulting pellet was resuspended and incubated for 1 h at room temperature with membrane washing buffer (10 mM Hepes/NaOH, pH 7.4, 0.5 M NaCl, 0.1 mM *p*-chloromercuriphenyl sulfonic acid, 5 mM EDTA, 0.25% trasylol, 5 mM NaF, 10 mM benzamidine/HCl, 0.1 mM Na_3VO_4, 0.2 mM PLP, 3 mM PMSF), followed by ultracentrifugation at 100,000 *g* for 1 h to pellet the washed membranes. Washed membranes were extracted for 2 h at 4°C with hypotonic Hepes buffer containing 1% Triton X-114 followed by centrifugation for 45 min at 100,000 *g* to separate the particulate extract (P) from insoluble debris. The cytosol proteins and proteins extracted from washed membranes were subjected to Triton X-114 partition assays.

Triton X-114 partitioning assays were performed on the subcellular fractions described above or on Triton X-114 detergent extracts of total cells, using a modification of the procedure of Bordier (1981). For comparative analyses of the amphiphilic properties of GAD_{65} in subcellular fractions, buffer compositions were adjusted to achieve identical conditions in each fraction. Triton X-114 phase transition of each fraction was induced by incubation at 37°C for 2 min. Aqueous (A) and detergent (D) phases were separated by centrifugation at 12,000 *g* for 2 min. The detergent and aqueous phases were analyzed by 10% SDS-PAGE followed by immunoblotting according to Christgau et al. (1991, 1992) using antiserum 1266 or 1267 (dilution 1:1,000) for GAD_{65} and its mutant proteins and Ab-1 (dilution 1:500) for $p21^{H\text{-}ras}$ and its mutant proteins.

Immunofluorescence Analyses

COS cells seeded in cover slips were cultured to 60–70% confluency before transfection with a GAD_{65} expression construct or a control plasmid. 20 h later the cells were processed for immunofluorescence analysis. Each manipulation was preceded by washing of cells three times in PBS. Cells were fixed in methanol for 5 min at −20°C, before incubation in blocking buffer (PBS, 2.5% fetal bovine serum, 0.02% azide) for 10 min at room temperature. The cells were incubated with the 1701 antiserum (diluted 1:500 in blocking buffer) for 30 min at room temperature followed by incubation with a secondary antibody (rhodamine conjugated goat anti-rabbit antiserum; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:100 in blocking buffer. After the final wash, the cover slips were inverted onto slides with mounting medium (1 mg/ml phenylenediamine in 10 mM Tris-HCl pH 8.5, 90% glycerol), sealed with nail polish, and observed through a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY).

Results

Palmitoylation and Membrane Anchoring Are Intrinsic Properties of the NH_2-terminal Region of GAD_{65}

Analyses of trypsin fragments of GAD_{65} have suggested that palmitoylation and membrane anchoring reside in a small NH_2-terminal fragment of GAD_{65} (Christgau et al., 1992). To confirm this finding, a NH_2-terminal deletion mutant of GAD_{65}, lacking the first 101 amino acids ($GAD_{65}\Delta$1-101), was generated and analyzed for its palmitoylation and membrane anchoring properties by transient expression in COS-7 cells. Wild-type (wt) GAD_{65} incorporates [^{3}H]palmitic acid in COS-7 cells (Fig. 1, lane *1*) and becomes membrane anchored. In contrast the $GAD_{65}\Delta$1-101 mutant is not palmitoylated (Fig. 1, compare lanes *5* and *10*). Furthermore, immunoblotting analysis of soluble and particulate fractions isolated from transfected COS-7 cells detected the $GAD_{65}\Delta$1-101 protein exclusively in soluble fractions (results not shown). Those results provide strong evidence that palmitoylation and membrane anchoring is mediated by the NH_2-terminal 101 amino acids of GAD_{65}.

Site-directed Mutagenesis of Cys 30 and Cys 45 Abolishes Palmitoylation of the GAD_{65} Molecule

The palmitoylation in the NH_2-terminal domain of GAD_{65} is hydroxylamine sensitive suggesting a thioester linkage (Christgau et al., 1992). To localize the site(s) of palmitoyla-

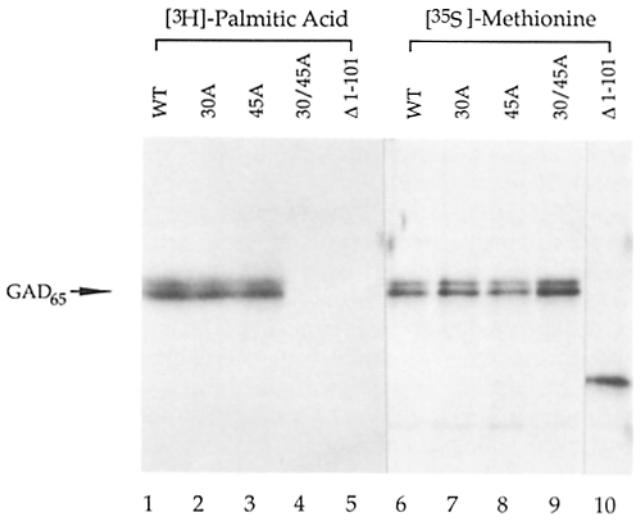

Figure 1. Site-directed mutagenesis of Cys 30 and Cys 45 abolishes palmitoylation in GAD_{65}. SDS-PAGE of wt GAD_{65} and mutant proteins labeled with [^{3}H]palmitic acid or [^{35}S]methionine. COS-7 cells transiently expressing wt GAD_{65}, or the 30A, 45A, Δ1-101, and 30/45A mutant proteins were labeled with either [^{3}H]palmitic acid (lanes *1–5*) or [^{35}S]methionine (lanes *6–10*) and immunoprecipitated with either the GAD6 antibody (lanes *1–4* and *6–9*) or the 1266 antiserum (lanes *5* and *10*). 30A: replacement of Cys 30 with Ala; 45A: replacement of Cys 45 with Ala; Δ1-101: deletion of the NH_2-terminal 101 amino acids; 30/45A: replacement of both Cys 30 and Cys 45 with Ala. To obtain similar intensity of radioactive proteins on fluorograms, the ratio between immunoprecipitated [^{35}S]methionine and [^{3}H]palmitic acid–labeled proteins subjected to SDS-PAGE was 1:40.

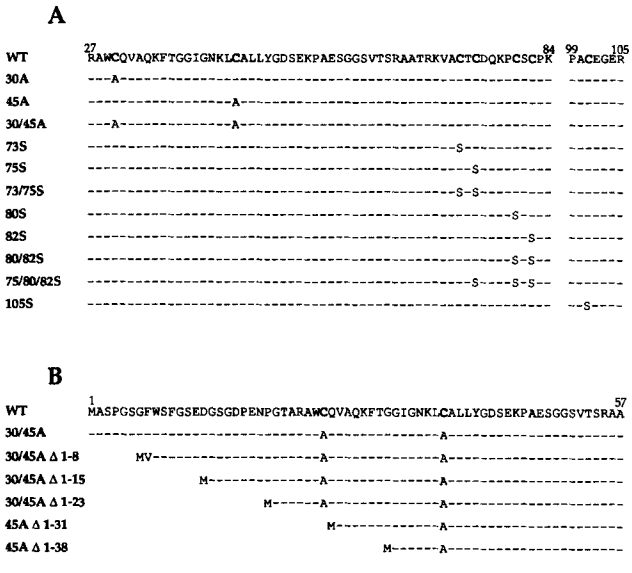

Figure 2. Sequence of Cys substitution mutants and NH_2-terminal deletion mutants of GAD_{65}. (*A*) Mutations of cysteine residues in the NH_2-terminal region of GAD_{65}. The wt GAD_{65} sequence is given at the top with the positions of the cysteines to be mutated given in bold face. The substitutions for these cysteines are indicated for each mutant. The dashed lines denote amino acids which are identical in wt and mutant proteins. (*B*) NH_2-terminal deletions of the GAD_{65}30/45A mutant. The wild-type sequence is indicated at the top. The dashed lines indicate amino acids which are identical in wt and mutant protein. None of the deletion mutants carry the palmitoylation sites (Cys 30 and Cys 45). An additional substitution of Phe to Val was introduced into the Δ1-8 deletion mutant to generate a Kozak DNA consensus sequence for translation efficiency (Kozak 1991).

tion and to investigate the role of this fatty acid in membrane anchoring, cysteine residues in the NH_2-terminal region were systematically substituted by alanine or serine residues using site directed in vitro mutagenesis (Fig. 2 *A*). The substitution mutants were transiently expressed in COS-cells and analyzed for incorporation of [^{3}H]palmitic acid in biosynthetic labeling experiments. All of the single cysteine substitution mutants incorporated [^{3}H]palmitic acid (not shown). Analyses of a series of mutants containing substitutions of combinations of two and three cysteines in the NH_2-terminal region (Fig. 2 *A*) identified one mutant that had lost the ability to incorporate [^{3}H]palmitic acid (Fig. 1, lane *4*). All the other combinations of double and triple cysteine mutations shown in Fig. 2 *A* incorporated palmitic acid (not shown). In the non-palmitoylated mutant (30/45A) Cys 30 and Cys 45 were replaced by alanine. The result suggests that Cys 30 and Cys 45 are the sites of posttranslational palmitoylation in GAD_{65}.

Non-palmitoylated Form of GAD_{65} Remains Hydrophobic and Membrane Bound in COS-7 Cells

To investigate the effect of loss of palmitoylation on the hydrophobicity of GAD_{65}, Triton X-114 extracts of COS-7 cells expressing either wt GAD_{65} or the 30/45A mutant were subjected to temperature induced Triton X-114 phase transition. The detergent and aqueous phases were separated

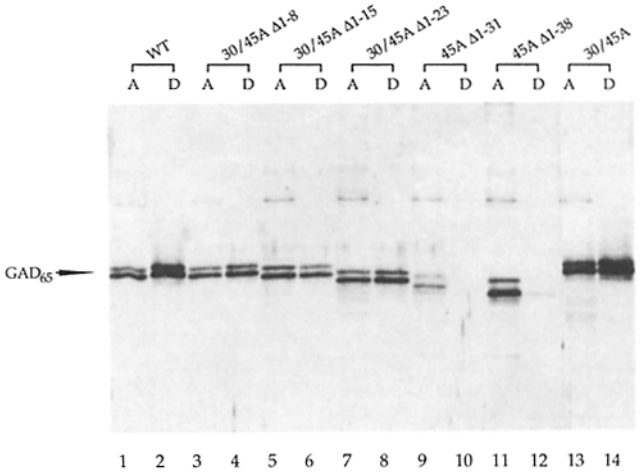

Figure 3. Partition of GAD_{65}, GAD_{65}30/45A and deletion mutants of GAD_{65}30/45A into Triton X-114 detergent phase. Triton X-114 total cell extracts of COS-7 cells expressing the indicated mutants were subjected to temperature induced phase separation and the aqueous (*A*) and detergent (*D*) phases analyzed by SDS-PAGE and immunoblotting using the 1267 antiserum, which recognizes the COOH terminus of GAD.

and analyzed by immunoblotting (Fig. 3). These analyses showed that the 30/45A mutant partitioned into the Triton X-114 detergent phase at a similar ratio as the wt protein (Fig. 3, compare lanes *1* and *2* with lanes *13* and *14*) demonstrating that the non-palmitoylated 30/45A mutant is still amphiphilic. This result is consistent with the finding that palmitoylation is not the first step in the posttranslational hydrophobic modification of GAD_{65}, but rather a subsequent step that distinguishes the hydrophobic soluble form, which has undergone the first modification, from a hydrophobic membrane anchored form (Christgau et al., 1991, 1992).

To assess the role of palmitoylation in membrane anchoring of the GAD_{65} molecule, we analyzed the subcellular distribution of wt and the non-palmitoylated GAD_{65}30/45A mutant expressed in COS-7 cells. The distribution of GAD_{65} in those experiments was compared with wt and a non-palmitoylated mutant of $p21^{H\text{-}ras}$ which were co-transfected into COS-7 cells and analyzed in parallel.

Palmitoylation of Cys 181 and Cys 184 in $p21^{H\text{-}ras}$ is required for membrane anchoring and mutation of these residues to serines ($p21^{H\text{-}ras}$181/184S) abolishes membrane anchoring (Hancock et al., 1990). COS-7 cells co-transfected with either wt GAD_{65} and wt $p21^{H\text{-}ras}$ (Fig. 4, lanes *1–4*) or GAD_{65}30/45A and $p21^{H\text{-}ras}$181/184S (Fig. 4, lanes *13–16*) were homogenized and separated into a cytosol and a particulate membrane fraction. The membrane fraction was washed in 0.5 M NaCl. Proteins in the cytosolic and washed membrane fractions were subjected to Triton X-114 phase separation. The distribution of wt and non-palmitoylated mutants of GAD_{65} and $p21^{H\text{-}ras}$ into the detergent and aqueous phases of each fraction was analyzed by immunoblotting. The results of these analyses are shown in Fig. 4, lanes *1–4* and *13–16*.

$p21^{H\text{-}ras}$ is synthesized as a hydrophilic soluble molecule (Fig. 4, lanes *1* and *13*) and undergoes a two step posttranslational modification at its carboxyl terminus to become membrane anchored. The first step involves farnesylation of Cys

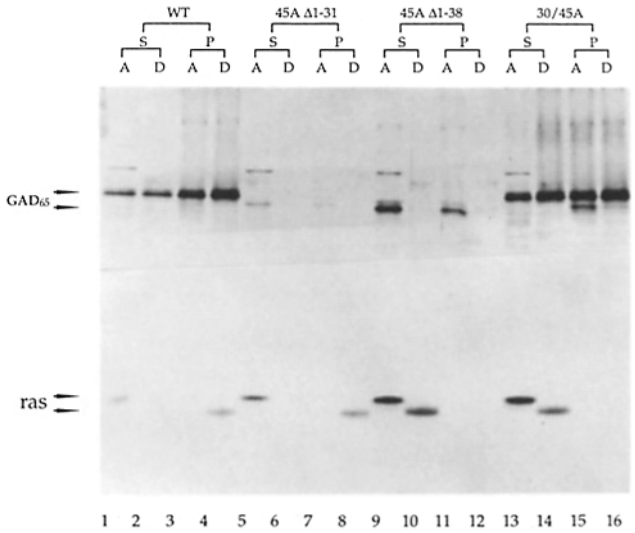

Figure 4. Subcellular distribution of GAD_{65}, GAD_{65}30/45A, and deletion mutants of GAD_{65}30/45A in COS-7 cells. A cytosolic fraction (*S*) and an extract of a washed membrane fraction (*P*) were prepared from COS-7 cells expressing the indicated proteins and subjected to Triton X-114 phase separation. Wild-type $p21^{H\text{-}ras}$ was co-expressed with wt GAD_{65} (lanes *1–4*) and the GAD_{65}30/45AΔ1-31 mutant (lanes *5–8*). The non-palmitoylated ras mutant 181/184S was co-expressed with the GAD_{65}30/45AΔ1-38 (lanes *9–12*) and GAD_{65}30/45A (lanes *13–16*). Aqueous (*A*) and detergent (*D*) phases were analyzed by SDS-PAGE and immunoblotting with the antiserum 1267 for GAD_{65} and its mutants and antiserum Ab-1 for $p21^{H\text{-}ras}$ and its mutants. Arrows indicate the position of the GAD and ras proteins. The wild-type ras is detected as an immature cytosolic form present only in the aqueous phases (lanes *1* and *5*) and as the fully modified farnesylated and palmitoylated membrane anchored form detected exclusively in the detergent phase (lanes *4* and *8*). In the 181/184S mutant of ras, the palmitoylation sites (Cys 181 and Cys 184) are mutated to serine. Lack of palmitoylation abolishes membrane anchoring and hence this mutant accumulates as the immature hydrophilic and cytosolic form (lanes *9* and *13*) and as a farnesylated, hydrophobic but soluble form (lanes *10* and *14*). In contrast, lack of palmitoylation of the GAD_{65} protein does not result in a significant change in membrane anchoring or distribution between aqueous and detergent phases (compare lanes *1–4* with lanes *13–16*). However deletion of amino acids 1–31 and 1–38 in the non-palmitoylated GAD_{65}30/45A mutant result in hydrophilic mutant proteins predominantly located in the cytosol.

186, proteolytic removal of the last three amino acids and carboxyl methylation of the Cys 186 carboxyl terminus (Hancock et al., 1989; Schafer et al., 1989; Casey et al., 1989; Gutierrez et al., 1989). The farnesylated form of ras is hydrophobic and cytosolic. The second step involves a reversible palmitoylation of Cys 181 and Cys 184 and results in a firmly membrane anchored protein (Hancock et al., 1989) (Fig. 4, lane *4*). In the $p21^{H\text{-}ras}$181/184S mutant membrane anchoring is abolished (Hancock et al., 1990) and the farnesylated form of $p21^{H\text{-}ras}$ accumulates (Fig. 4, lane *14*).

In COS-7 cells, as in pancreatic β cells, wt GAD_{65} protein is detected in both cytosol and membrane fractions, consistent with its presence in both soluble and membrane anchored forms (Fig. 4, lanes *1–4*; Christgau et al., 1991, 1992). In both cytosolic and membrane fractions the wt GAD_{65} protein distributes into the Triton X-114 detergent phase consistent with the hydrophobic soluble and hydrophobic membrane anchored forms demonstrated for this protein (Christgau et al., 1991, 1992). The hydrophobic GAD_{65} forms, in contrast to the $p21^{H\text{-}ras}$ forms, never partition completely into the detergent phase but are consistently detected in both aqueous and detergent phases (Christgau et al., 1991, 1992). It is likely that the size of GAD_{65} precludes its quantitative partitioning into the detergent phase (Bjerrum et al., 1983). The majority of the wt GAD_{65} protein is however found together with the wt $p21^{H\text{-}ras}$ protein in the detergent phases of the membrane fraction (Fig. 4, lane *4*). The GAD_{65}30/45A mutant behaved similarly to the wt protein in those analyses (Fig. 4, compare lanes *1–4* with *13–16*). Thus a significant fraction of the non-palmitoylated mutant protein was found in membrane fractions where it distributed into the Triton X-114 detergent phase (Fig. 4, lane *16*). In comparison, the $p21^{H\text{-}ras}$181/184S mutant was found exclusively in the cytosol fraction. Thus in contrast to $p21^{H\text{-}ras}$, palmitoylation of the GAD_{65} protein is not a requisite for membrane anchoring. Instead other modification(s) in the NH_2-terminal region are implied. Those results motivated an investigation of which part of the NH_2-terminal region is required for hydrophobic modification of membrane anchoring of GAD_{65}.

Amino Acid Residues 24–31 Are Essential for Membrane Anchoring of GAD_{65}

To further localize the sequences in the NH_2-terminal region which are required for targeting and anchoring of GAD_{65} to membrane compartments, we analyzed the effect of stepwise deletions of NH_2-terminal sequences in the GAD_{65}30/45A mutant on the hydrophobicity and membrane anchoring of the resulting proteins (Fig. 2 *B*). The mutants were transiently expressed in COS-7 cells and analyzed in Triton X-114 partition assays (Fig. 3), in parallel with wt GAD_{65} and the 30/45A mutant from which they were derived. Deletions of up to 23 NH_2-terminal amino acids of the 30/45A mutant did not result in significant changes in hydrophobicity of the resulting proteins (Fig. 3, compare lanes *3–8* with lanes *1–2*). However, deletion of an additional eight amino acids (Δ1-31) resulted in a protein which did not partition into the Triton X-114 detergent suggesting that this protein does not undergo hydrophobic modifications and hence remains hydrophilic. This mutant protein was expressed at lower levels than the shorter deletion mutants (Figs. 3 and 4) suggesting that it is less stable than the other mutants. However deletion of an additional seven amino acids (Δ1-38) resulted in a protein which was expressed at similar levels as the shorter deletion mutants. The Δ1-38 mutant also did not partition into the Triton X-114 detergent phase demonstrating that it does not undergo hydrophobic modification and therefore remains hydrophilic (Fig. 3). Analyses of subcellular distribution of the deletion mutants in COS-7 cells confirmed that both the Δ1-31 and Δ1-38 mutants were hydrophilic and predominantly localized in the cytosol fraction (Fig. 4, lanes *5* and *9*). In conditions (wash of membrane fraction in 0.5 M NaCl) where all of the non-palmitoylated but farnesylated $p21^{H\text{-}ras}$ 181/184S protein was localized in the cytosolic fraction (Fig. 4, lane *10*), a small fraction of the non-palmitoylated and hydrophilic Δ1-31 and Δ1-38 GAD_{65} was detected in the aqueous phase of the membrane fraction (Fig. 4, lanes *7* and *11*). Thus it appears

that the Δ1-31 and Δ1-38 deletion mutants maintain a low level of membrane avidity in the absence of posttranslational hydrophobic modification(s) perhaps by association with a membrane protein. These data show that deletion of amino acids 24–31 in the non-palmitoylated GAD_{65} mutant results in a loss of hydrophobic modification and a predominant localization in the cytosol. Thus amino acids 24–31 are required for hydrophobic modification and appropriate membrane anchoring of GAD_{65}.

Immunofluorescence Analyses of Wild Type and Mutants of GAD_{65} in COS-7 Cells

GAD_{65} is anchored to the membrane of synaptic-like microvesicles in pancreatic β cells (Christgau et al., 1992), but remains associated with the Golgi apparatus in transfected CHO cells (Solimena et al., 1993). We analyzed the subcellular distribution of wt GAD_{65}, the 30/45A mutant, and the Δ1-38 mutant in COS-7 cells by indirect immunofluorescence. In COS-7 cells wt GAD_{65} is localized in a large membranous perinuclear compartment, and in punctate like structures that may represent membrane vesicles (Fig. 5 *a*). No significant difference was observed between the wt protein and the 30/45A mutant (not shown). The Δ1-38 mutant was evenly distributed in a diffuse pattern throughout the cytosol (Fig. 5 *b*), which is similar to the cellular distribution of GAD_{67} (Solimena et al., 1993; and our unpublished results). The immunofluorescence analyses of the cellular distribution of wt GAD_{65} and the two mutants are consistent with the results from subcellular analyses and confirm that the Δ1-38 deletion mutant is predominantly cytosolic in contrast to the wt protein which is associated with membrane compartments.

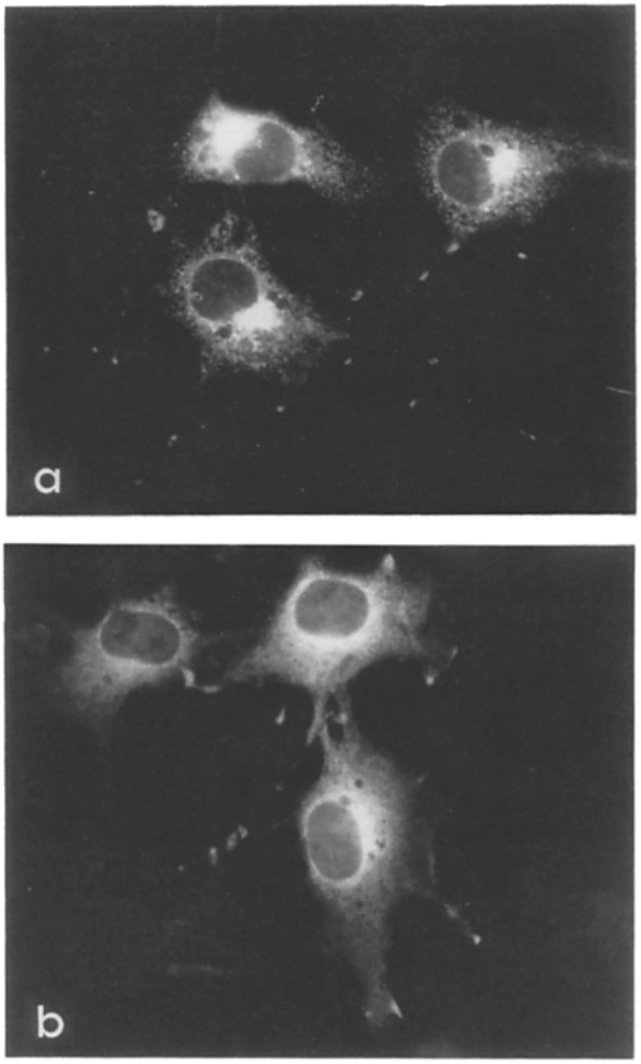

Figure 5. Immunofluorescence analyses of COS-7 cells expressing GAD_{65} and the deletion mutant GAD_{65}30/45AΔ1-38. COS cells transfected with wt GAD_{65} (*a*) and GAD_{65}30/45AΔ1-38 (*b*) cDNAs were immunostained with antiserum 1701 followed by visualization using a fluorescence conjugated goat anti-rabbit antiserum. The wt GAD_{65} is concentrated in a perinuclear compartment and in small punctate like structures (vesicles) in the cytoplasm. The non-palmitoylated deletion mutant of GAD_{65} shows diffuse cytoplasmic staining.

Discussion

The NH_2-terminal region distinguishes the two isoforms of mammalian glutamate decarboxylase, GAD_{65} and GAD_{67}, and is altogether absent in *Drosophila* GAD (Erlander et al., 1991). It is possible that this region has evolved to target the two mammalian enzymes to different sub-cellular compartments. Thus, while the GAD_{67} protein is localized in the cytosolic compartment, the NH_2-terminal region of GAD_{65} mediates targeting and anchoring to membranous compartments in both endocrine (Christgau et al., 1992) and non-endocrine cells (Christgau et al., 1992; Solimena et al., 1993).

Pulse–chase analyses of GAD_{65} in pancreatic β cells suggest that the newly synthesized hydrophilic enzyme is first modified to become a hydrophobic molecule which is cytosolic or of a low membrane avidity. This form then undergoes a second set of modifications that include a hydroxylamine sensitive palmitoylation in the NH_2-terminal region. These modifications result in a firmly membrane-anchored molecule which remains membrane anchored in conditions that release peripheral membrane proteins (Christgau et al., 1991). Because palmitoylation is a distinct property of the membrane anchored form (Christgau et al., 1992) we previously proposed that palmitoylation might be important for membrane anchoring of GAD_{65}. Palmitoylation is clearly required for membrane association of some of the ras proteins, where mutation of palmitoylated cysteines results in loss of membrane anchoring (Hancock et al., 1990). In this study we have identified Cys 30 and Cys 45 as the sites of palmitoylation in the GAD_{65} molecule by site-directed mutagenesis. Mutation of both of these residues abolishes palmitoylation of GAD_{65} in COS-7 cells. The non-palmitoylated mutant is still hydrophobic, which is consistent with a biosynthetic pathway in which the protein first undergoes an irreversible hydrophobic posttranslational modification(s) that results in a hydrophobic yet soluble molecule. Surprisingly, the non-palmitoylated GAD_{65}30/45A mutant is still membrane anchored in COS-7 cells, demonstrating that palmitoylation is not essential for membrane anchoring. Our results suggest that the reversible membrane anchoring of the GAD_{65} molecule (Christgau et al., 1992) is not mediated exclusively by a palmitoylation–depalmitoylation mechanism as it is in some of the ras proteins (Magee et al., 1987;

Hancock et al., 1989, 1990). Rather GAD_{65} undergoes additional posttranslational modification(s) that contribute to its membrane association. The nature of this modification is currently unknown, but our previous work indicates that several lipid modifications known to contribute to membrane anchoring, including polyisoprenylation, phosphatidylinositolglypidation or myristoylation, are not involved in anchoring GAD_{65} (Christgau et al., 1992).

Using the palmitoylation minus mutant of GAD_{65}, we have dissected the NH_2-terminal region by deleting segments of the NH_2 terminus and analyzing the hydrophobicity and membrane anchoring properties of the resulting mutants. The non-palmitoylated mutant lacking amino acids 1–23 was not significantly different from the wild-type protein with regard to hydrophobicity, subcellular localization and membrane anchoring. Thus the first 23 amino acids in the NH_2 terminus of the GAD_{65} molecule are not required for hydrophobic modification, subcellular targeting, and membrane anchoring. However, deletion mutants which lacked an additional 8 or 15 amino acids, respectively (aa 24–31, aa 24–38) failed to undergo hydrophobic modification(s) and were predominantly localized in the cytosol. Thus it appears that amino acids 24–31 are required for both the first hydrophobic modification and the subsequent membrane anchoring of GAD_{65}, suggesting that those events may be closely linked. It is conceivable that the first hydrophobic modification is a prerequisite for the subsequent membrane anchoring modification, as is the case for farnesylation and palmitoylation in $p21^{H\text{-}ras}$ and $p21^{N\text{-}ras}$ (Hancock et al., 1989). Alternatively, the first hydrophobic modification and the membrane anchoring moiety in GAD_{65} may be independent but spatially linked and thus both affected by deletion of amino acids 24–31. Mass spectrometric analyses will address the question whether the amino acid 24–31 segment itself is posttranslationally modified in the wild-type protein.

It can not be excluded that deletion of amino acids 24–31 in the GAD_{65} protein results in a conformational change elsewhere in the molecule which is incompatible with targeting and anchoring of GAD_{65} to membrane compartments. It is more likely however that the major effect of deletion is local, which would suggest that the palmitoylated Cys 30 and Cys 45 residues are adjacent to the additional hydrophobic and membrane anchoring moieties in GAD_{65}.

What is the role of palmitoylation in GAD_{65}? Our results suggest that palmitoylation is not required for membrane anchoring of GAD_{65}. One possibility is that palmitoylation presents a redundant or alternative membrane anchoring moiety in the GAD_{65} protein perhaps analogous to the situation in the α chain of several of the trimeric GTP-binding proteins, which are both palmitoylated and myristoylated (Linder et al., 1993; Parenti et al., 1993). Alternatively, it is possible that palmitoylation of GAD_{65} adjacent to the membrane anchoring region has a different role, which is apparent in certain other membrane proteins. For example, the Vesicular Stomatitis Virus G protein (Rose et al., 1984), CD4 (Crise and Rose, 1992), the transferrin receptor (Jing and Trowbridge, 1987), and the heavy chain of the human class I histocompatibility antigen HLA-B7 (Kaufman et al., 1984; Ploegh et al., 1981) each are palmitoylated at cysteines adjacent to their membrane spanning region. In those proteins palmitoylation is not necessary for stable membrane association. Rather palmitoylation may serve a different role. In the transferrin receptor, lack of palmitoylation increases the rate of endocytosis (Alvarez et al., 1990). In the β2-adrenergic receptor, palmitoylation seems to be important for coupling to the guanyl nucleotide regulatory protein G_s (Moffett et al., 1993). By analogy palmitoylation of Cys 30 and Cys 45 adjacent to the aa 23–31 segment required for membrane anchoring of GAD_{65} may be important for modifying the orientation or folding of the protein at the membrane of synaptic vesicles or synaptic like microvesicles. Such folding may facilitate protein–protein or protein–lipid interactions and be important for the function of the enzyme.

We have shown that a 57-kD hydrophilic soluble COOH-terminal fragment of GAD_{65} can be spontaneously released from islet cell membranes as a function of time suggesting an enzymatic reactivity that can cleave the protein downstream from the membrane anchoring region (Christgau et al., 1992). The 57-kD fragment is detected in islet cell (Christgau et al., 1991) and brain extracts (our unpublished results) suggesting that this reactivity is present in both tissues. Thus, in addition to the reversible membrane anchoring of GAD_{65}, which may confer a flexible and perhaps regulated on–off mechanism of membrane anchoring, there may be a second and irreversible mechanism that can release the protein from membrane compartments as a fragment that has lost the membrane anchoring domain.

It is not known whether the reversible targeting of GAD_{65} to the membrane of synaptic vesicles or synaptic-like microvesicles plays a role in its presentation to the immune system and thus its unique autoantigenicity compared with GAD_{67}. It can be hypothesized that reversible anchoring to the membrane of synaptic vesicles which are part of the endocytic/exocytic membrane network, and perhaps also the proteolytic release mechanism described above, targets the protein for increased degradation and presentation by MHC class I antigens for recognition by cytotoxic T-cells. Alternatively, the membrane-anchored GAD_{65} may be targeted to the surface during exocytosis for recognition by autoantibodies. Whereas the first mechanism appears relevant for T-cell–mediated destruction of pancreatic β cells, the second possibility may be the only mechanism by which GABA-ergic neurons can present the protein to the immune system in the absence of MHC class I antigen expression.

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